EXHIBIT 18

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Differential *N*-Glycan Patterns of Secreted and Intracellular IgG Produced in *Trichoplusia ni* Cells*

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Structures of the *N*-linked oligosaccharide attached to the heavy chain of a heterologous murine IgG_{2a} produced from *Trichoplusia ni* (TN-5B1-4, High Five) insect cells were characterized. Coexpression of the chaperone immunoglobulin heavy chain-binding protein (BiP) in the baculovirus-infected insect cells increased the soluble intracellular and secreted IgG level. This facilitated the detailed analysis of *N*-glycans from both intracellular and secreted IgG. Following purification of the immunoglobulins using Protein A-Sepharose, glycopeptides, prepared by trypsin-chymotrypsin digestion, were further digested with glycoamidase from sweet almond emulsin to obtain the oligosaccharide moieties. The resulting oligosaccharides were then reductively aminated with 2-aminopyridine and the structures identified by two-dimensional high performance liquid chromatography mapping (Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., and Takahashi, N. (1988) *Anal. Biochem.* 171, 73–90). The *N*-glycans obtained from the secreted IgG contain 35% complex type, some with terminal galactose residues at either α1,3-Man or α1,6-Man branches of the $Man_3GlcNAc_2$ core. The remaining oligosaccharides detected in the secreted IgG were principally hybrid (30%) and paucimannosidic (35%) type *N*-glycans. Most (84%) of these secreted glycoforms contained fucose α1,6-linked to the innermost GlcNAc residue and the presence of a potentially allergenic fucose α1,3-linked to the innermost GlcNAc residue was also detected. In contrast, the intracellular immunoglobulins included 50% high mannose-type *N*-glycans with lower levels of complex, hybrid, and paucimannosidic-type structures. Reverse phase one-dimensional high performance liquid chromatography analysis of the IgG *N*-glycans in the absence of heterologous BiP exhibited a similar distribution of intracellular and secreted glycoforms. These studies indicate that *Trichoplusia ni* TN-5B1-4 cells are capable of terminal galactosylation. However, the processing pathways in these cell lines appear to diverge from mammalian cells in the formation of paucimannosidic structures, in the presence of α1,3-fucose linkages, and in the absence of sialylation.

Insect cells have been widely utilized as hosts for the production of numerous glycoproteins through the baculovirus expression system (1, 2). Native insect cell glycoproteins also serve as models for developmental processes in eucaryotes (3, 4). While the structure, synthesis, and function of oligosaccharides in mammalian glycoproteins are well characterized, information on the carbohydrate structures and processing pathways present in insect cells is limited and sometimes contradictory (5–7). The oligosaccharides in glycoproteins can play critical roles in cellular targeting, structural stability, resistance to proteolysis, immunogenicity, and circulatory half-life (8, 9). With insects representing more than half of the animal species classified (5), there is a need to obtain more information on the carbohydrate structures and processing of glycoproteins from insect cells.

Many initial studies of *N*-glycans in insect cell-derived heterologous glycoproteins indicated the presence of only high mannose-type or short truncated, paucimannosidic oligosaccharides,[1] sometimes containing L-fucosyl[2] residues (6, 7). These observations confirmed the earlier studies of endogenous insect cell glycoproteins which were similarly found to lack complex carbohydrate structures (10–12). It was presumed that insect cells did not possess the capacity to synthesize complex-type oligosaccharides since the levels of sialyl-, galactosyl-, and *N*-acetylglucosamine transferases were found to be insignificant (12).

In contrast, studies with the recombinant human plasminogen indicated that insect cells could synthesize complex *N*-linked oligosaccharides (13). Several more recent studies on homologous glycoproteins also have indicated that certain insect cell lines can synthesize hybrid and complex oligosaccharides. Honeybee venom phospholipase A_2 (PLA_2)[3] was found to

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[1] In this study, high mannose-type *N*-linked oligosaccharides refers to structures $Man_{4-9}GlcNAc_2$. Paucimannosidic structures contain $Man_{3-2}GlcNAc_2$ with or without attached fucose residues at the innermost GlcNAc residue. Hybrid structures include a terminal GlcNAc, Gal, sialic acid, or another non-Man residue attached to either the α1,3- or α1,6-Man branch of the $Man_3GlcNAc_2$ core. Complex structures include a terminal GlcNAc, Gal, sialic acid, or another non-Man residue attached to both α1,3- and α1,6-Man branches.

[2] All sugars are of D-configuration except for fucose which is L-configuration.

[3] The abbreviations used are: PLA_2, phospholipase A_2; PA, pyridylamino; BiP, immunoglobulin heavy chain-binding protein; ESI, electrospray ionization; JPLH, recombinant baculovirus containing IgG heavy and light chain genes; AcBB-BiP, recombinant baculovirus containing BiP gene; IgG-E, an *N*-acetyllactosamine-type oligosaccharide from human IgG; PLA-9, oligosaccharide from honeybee venom PLA_2; BG60-B, oligosaccharide from Bermuda grass antigen BG60; NS/0, murine NS/0 cell line; GlcNAc-TII, *N*-acetylglucosamine transferase II; α-Man II, α-mannosidase II; β1,4-GalT, β1,4-galactose transferase.

contain a terminal fucosylated GalNAcβ1–4GlcNAc (LacdiNAc) moiety (14, 15) and apolipophorin III from *Locusta migratoria* included terminal GlcNAc residues substituted with 2-aminoethylphosphonate. Analysis of the native membrane glycoproteins from three insect cell lines, *Mamestra brassicae* (MB-0503), *Spodoptera frugiperda* (Sf21), and *Bombyx mori* (Bm-N), revealed the presence of a small percentage of hybrid *N*-glycans with GlcNAc at the reducing termini (5). Some of these structures were difucosylated with both α1,3- and α1,6-linkages at the innermost GlcNAc residue. The presence of sialic acids has also been reported for recombinant glycoproteins secreted from *Trichoplusia ni* larvae (16). Some of these structural features of *N*-linked oligosaccharides were further justified by the detection of GlcNAc transferase I and II (17, 18), fucosyltransferase (17), and mannosidases I and II (19–21).

Analysis of *N*-glycans of heterologous interferon-γ secreted from *Estigmena acrea* (Ea-4) and *S. frugiperda* (Sf-9) cell lines using HPLC and mass spectrometry confirmed that hybrid and complex *N*-glycans could be obtained from heterologous proteins produced by insect cells (22). Although the Ea-4 cells were observed to generate multiple complex and hybrid glycoforms, the Sf9 cells were observed to produce only hybrid *N*-glycans.

We felt that an examination of the carbohydrate structures obtained from both intracellular and secreted sources would provide further insights into the oligosaccharide processing pathways present in insect cell lines. Therefore, the *N*-glycans of a heterologous immunoglobulin G produced by baculovirus-infected insect cells were characterized using two-dimensional HPLC analysis of 2-aminopyridine-modified oligosaccharides (23). The immunoglobulin was purified from the cell lysate and culture medium of baculovirus-infected *T. ni* (TN5-B1–4, High Five) cells, a popular host cell line for the production of heterologous proteins. To generate sufficient soluble intracellular and secreted immunoglobulin for subsequent oligosaccharide analysis, coexpression of the chaperone, immunoglobulin heavy chain-binding protein (BiP), was utilized. This chaperone has been shown to prevent immunoglobulin aggregation and enhance soluble intracellular and secreted IgG levels from baculovirus-infected insect cells (24–26). Subsequent one-dimensional HPLC analysis of the *N*-glycans in the absence of heterologous BiP indicated that the coexpression of BiP did not significantly alter the particular glycoforms observed. A comparison of the carbohydrates from intracellular and secreted IgG indicated significant structural differences between *N*-glycans obtained from the two sources and helped to elucidate further the *N*-linked oligosaccharide processing pathway in insect cells. Furthermore, a comparison of the *N*-glycans with those produced in mammalian cells can illustrate the similarities and differences that exist in insect and mammalian processing pathways.

EXPERIMENTAL PROCEDURES

Materials

T. ni insect cells (BTI-TN5B1-4, known commercially as High Five) were obtained from Invitrogen (Portland, OR) and grown in ExCell 401 or ExCell 405 medium from JRH Biosciences (Lenexa, KS). The medium was supplemented with 5 mM glutamine/penicillin-streptomycin from Life Technologies, Inc. (Gaithersburg, MD). The Sephadex G-15, Protein A-Sepharose beads, column, and fast protein liquid chromatography are products of Pharmacia Biotech Inc. (Uppsala, Sweden). Phosphate-buffered saline, Triton X-100, Tris, iodoacetamide, phenylmethylsulfonyl fluoride, trypsin, and chymotrypsin were purchased from Sigma. The membrane tubing was supplied by Spectrum Medical Industries, Inc. (Houston, TX). Glycoamidase A (EC 3.5.1.52, from sweet almond, commercially available as glycopeptidase A), β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase (jack bean) were purchased from Seikagaku Kogyo (Tokyo). α-L-Fucosidase (bovine kidney) was purchased from Boehringer Mannheim (Mannheim, Germany). Pronase was obtained from Calbiochem (La Jolla, CA). The pyridylamino (PA) derivatives of isomalto-oligosaccharides (4–20 glucose residues) and of *N*-linked oligosaccharides (code number M3.3, M5.1, M6.1, M7.1, M7.2, M8.1 M9.1, 000.1, 010.1, 100.2, 110.1, 110.2, 200.3, 210.1, 210.2)[4] were purchased from Nakano Vinegar Co. (Handa, Japan). The following materials were purchased from the sources indicated: Dowex 50W-X8 (H^+) and Dowex 1 (CO_3^{2-}), from Dow Chemical Co. (Midland, MI); sodium cyanoborohydride, Aldrich; 2-aminopyridine, Wako Pure Chemical Industries (Osaka, Japan).

Methods

Baculovirus Expression Vector and Cell Culture—Recombinant baculovirus, JPLH, containing genes for the heavy and light chains of a mouse IgG_{2a} under the control of separate polyhedrin promoters, was kindly provided by Domdey (27). This IgG, directed against lipoprotein I of *Pseudomonas aeruginosa*, has been secreted as a fully assembled functional H_2L_2 complex including attached carbohydrates (24–26). One *N*-linked glycosylation site was found on the constant region of the heavy chain coded by JPLH.[5] *N*-Linked glycosylation sites were not observed on the light chain. Recombinant baculovirus coding for murine BiP, AcBB-BiP, has been described (24). *T. ni* (BTI-TN5B1-4, High Five) (27) insect cells were maintained at 27 °C in 150-ml shaker flasks containing 30 ml of culture medium (ExCell 401 or ExCell 405) rotated at 120 rpm. For IgG production, cells were maintained and infected in 1.0-liter shaker flasks (28).

Production and Purification of Mouse IgG_{2a} from Insect Cell Culture—High Five insect cells were grown in a shaker flask to 3×10^6 cells/ml and then subcultured into 300 ml of culture medium in two separate shaker flasks. These were infected at a cell density of 1×10^6 cells/ml with the baculovirus JPLH with and without AcBB-BiP at a multiplicity of infection of 5 for each virus. This multiplicity of infection was used to ensure simultaneous infection of all cells by two different viruses. One hundred ml of fresh medium was added to supplement the partially spent medium at 0, 24, and 48 h post-infection.

At 72 h post-infection, cells were separated from culture supernatant by centrifugation at 3,000 rpm for 5 min using a Beckman JR-21 centrifuge. IgG was then purified separately from the cell culture supernatant and cell lysates. Intact cells were rinsed with phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 20 mM iodoacetamide, pH 7.5) containing 1 mM freshly added phenylmethylsulfonyl fluoride. Both supernatant and cell lysates were clarified at 20,000 rpm for 20 min using the JR-21 centrifuge at 4 °C. Clarified samples were loaded onto a 10-ml Protein A-Sepharose column on an fast protein liquid chromatography at a flow rate of 1 ml/min. Bound IgG was eluted with 20 ml of elution buffer (50 mM acetic acid, 15 mM NaCl, pH 3.1) and the pH was adjusted to neutral immediately with 1 ml of 1 M Tris buffer (pH 7.5), in the collecting tube. Samples were dialyzed against 4 liters of deionized water in membrane tubing (M_r cut-off 12,000–14,000) at 4 °C with 3 changes of water. Dialyzed samples were then lyophilized.

Preparation and Derivatization of N-Linked Oligosaccharides from Insect IgG—Supernatant IgG (15 mg of protein correspond to 200 nmol of oligosaccharides) and lysate IgG (8 mg of protein) were heated at 100 °C for 10 min. After digestion of IgG glycoproteins with trypsin and chymotrypsin (each 1%, w/w, of the substrate protein), at pH 8.0, the peptide/glycopeptide mixture was treated with glycoamidase A (0.5–1 milliunit) in 30 μl of 0.5 M citrate/phosphate buffer at pH 4.0 for 16 h to release the oligosaccharides. Finally, the mixture was digested with 1% (w/w) of Pronase to convert peptidic materials to amino acids and small peptides. The oligosaccharide fraction was purified by successively passing through columns (1 ml for each) of Dowex 50W-X8(H^+) and Dowex 1(CO_3^{2-}) (29). The filtrate was evaporated to dryness and reductively aminated with a fluorescent reagent, 2-aminopyridine, and sodium cyanoborohydride (30). PA-oligosaccharides were purified by gel filtration on a Sephadex G-15 column with 10 mM ammonium bicarbonate. The derivatization of sialyl oligosaccharides with 2-aminopyridine was quantitative and did not affect the sialic acid content.

Isolation and Characterization of PA-Oligosaccharides by Three Successive HPLC Steps—The PA-oligosaccharide mixture was first separated on a TSKgel DEAE-5PW (7.5 × 75 mm, Tosoh) column according to the sialic acid content. Elution was performed at a flow rate of 1.0 ml/min at 30 °C using the two solvent components, A and B, as de-

[4] The structures of reference compounds are shown in Table I and Fig. 3. A complete list of code numbers and the corresponding structures are found in Ref. 23.

[5] H. Domdey, personal communication.

scribed previously (29). Each oligosaccharide separated by the DEAE column was evaporated *in vacuum* without desalting and directly injected onto an ODS-silica column (Shimpack CLC-ODS, 6 × 150 mm, Shimadzu). All the subsequent experimental procedures including chromatographic conditions have been reported previously (31). The elution volumes expressed as glucose units (using PA-derivatives of isomaltooligosaccharides) are used as X-coordinates. The individual peak from the ODS column was applied to an amide-silica column (TSKgel Amide-80, 4.6 × 250 mm, Tosoh), and the elution volumes expressed as glucose units were used as Y-coordinates. Plotting all the X- and Y-coordinates constitutes the two-dimensional map, from which the structures of unknown oligosaccharides could be estimated by comparing their coordinates with those of the standard compounds of known structures.

Exoglycosidase Digestion Procedure—Each of the PA-oligosaccharides (50 pmol) isolated from the ODS and the amide columns was sequentially digested with exoglycosidases (β-galactosidase, β-*N*-acetylhexosaminidase, and α-L-fucosidase) under the conditions described previously (23). Sialidase from *Arthrobactor ureafaciens* (20 milliunits) was used to treat 500 pmol of the substrate in 15 μl of 0.1 M citrate/phosphate buffer, pH 5.0, at 37 °C for 16 h (29).

^1H NMR Measurements—PA-oligosaccharide G (3 nmol) isolated by HPLC was desalted by gel filtration on a Sephadex G-15 column prior to NMR measurement. Characterization of linkage positions was with ^1H NMR performed on a Bruker AM 400 spectrometer (400-MHz) as described previously (29).

Mass Spectrometry—ESI (electrospray ionization) mass spectrum was measured with a double-focusing mass spectrometer JMS HX-110 (JEOL, Japan) equipped with an ESI ion source (Analytica of Branford). The mass spectrometer was operated at an acceleration voltage of 5 kV. The mass spectrum was acquired with a mass resolution of 1000 in the range of m/z 10,000–2000 by scanning a magnetic field in 22 s. The capillary tube was heated to 85–90 °C to assist in the evaporation of solvent from the droplets. The capillary skimmer voltage was set to 150 V. Sample was dissolved in methanol/water (3:1, v/v) containing 1% acetic acid at a concentration of 5 pmol/μl, and infused into the ESI ion source at a flow rate of 1 μl/min by a Harvard syringe pump (Harvard Apparatus). Mass spectrum was processed with JEOL DA7000 data system.

RESULTS

Purification of IgG from Cell Lysates and Culture Media—The High Five insect cells used in this study were infected with JPLH, coding for IgG_{2a}, in the presence or absence of AcBB-BiP. Heterologous IgG was purified from the culture supernatant and soluble cell lysates using a Protein A-Sepharose column. Co-infection of High Five cells with JPLH and AcBB-BiP increased the amount of the soluble immunoglobulin purified from the culture supernatant and cell lysate by a factor of 4 and 8, respectively (data not shown). Since the coexpression of heterologous BiP provided much higher levels of soluble intracellular IgG, subsequent two-dimensional HPLC oligosaccharide analysis was performed on immunoglobulin samples from cells coinfected with JPLH and AcBB-BiP.

Examination of the IgG samples from the JPLH + AcBB-BiP infection on SDS-10% polyacrylamide gel electrophoresis (Fig. 1) using nonreducing conditions revealed a prominent band corresponding to IgG purified from culture supernatant (*lane 1*) and cell lysate (*lane 2*). The identity of this protein as IgG with functional activity has been confirmed in prior immunoprecipitation, Western blot, and enzyme-linked immunosorbent assay studies (24–26).

Oligosaccharides of IgG Produced by Insect Cells—More than 90% of the total carbohydrate of both supernatant and lysate IgGs was released as reducing oligosaccharides by the sequential digestion of the IgGs with protease and glycoamidase A, as analyzed by the orcinol-H_2SO_4 reagent (32) (data not shown).

No acidic oligosaccharide was detected in the lysate IgG, but about 8% of the oligosaccharides from the supernatant IgG were retained on the DEAE column. The acidity, however, was shown not to arise from sialic acid. When the acidic fractions were treated with neuraminidase from *A. ureafaciens*, there was no release of sialic acids. ODS chromatography of the acidic fractions resulted in several minor components different from the known sialylated oligosaccharides including those from human and murine IgGs. We did not continue to investigate these minor components because of their limited quantities.

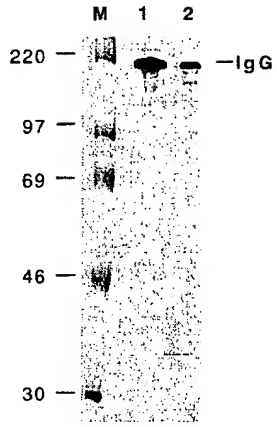


FIG. 1. **IgG purified from culture supernatant and cell lysate for insect cells infected with JPLH and AcBB-BiP.** Purified samples were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. *Lane 1*, samples purified from cell culture supernatant. *Lane 2*, samples purified from cell lysate. Molecular weight marker is indicated as *M*.

The PA-oligosaccharides derived from the supernatant and lysate IgGs were separated into 6 and 10 fractions, respectively, by reverse phase-HPLC on the ODS-silica column (Fig. 2). The fractions labeled A-L were collected separately and each was further fractionated by size using the amide-silica column. Oligosaccharide B was separated into B1 and B2, and oligosaccharide H was separated into H-1, H-2, and H-3, on the amide column (data not shown).

Identification of Each Oligosaccharide Structure—The structures of all unknown oligosaccharides were determined by comparing their positions with those of the known standard oligosaccharides on the two-dimensional map (containing more than 400 reference oligosaccharides). Identification of a sample PA-oligosaccharide was confirmed by co-chromatography with a known PA-oligosaccharide on the ODS and amide-silica columns. For further confirmation, the sample PA-oligosaccharide and the standard PA-oligosaccharide were digested in parallel with several exo-glycosidases (α-L-fucosidase, β-galactosidase, and β-*N*-acetylhexosaminidase for supernatant and lysate IgG oligosaccharides, and α-mannosidase for lysate oligosaccharides). At each step of the trimming, the elution positions of the resultant PA-oligosaccharide were shown to be identical with the corresponding product from the standard PA-oligosaccharide, with the exception of oligosaccharide G (see below). The trimming and comparison was continued until both sample and standard PA-oligosaccharides yielded the common trimannosyl core, Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc. The detailed structures of the oligosaccharides are shown in Table I.

Structure of Oligosaccharide G—The elution coordinates of oligosaccharide G did not coincide with that of any known standards. Although digestion with β-galactosidase or β-*N*-acetylhexosamidase did not change its coordinates, digestion with α-L-fucosidase (bovine kidney), known to digest Fucα1–6GlcNAc-PA but not Fucα1–3GlcNAc-PA, changed its coordinates from 8.5, 5.5 to 5.9, and 5.1 (Fig. 3). This pronounced change indicated the existence of a fucose residue linked to the PA-modified GlcNAc residue (GlcNAc-1) with an α1,6-linkage (33). The elution coordinates of this enzymatically de-α1,6-fucosylated oligosaccharide also did not correspond to any

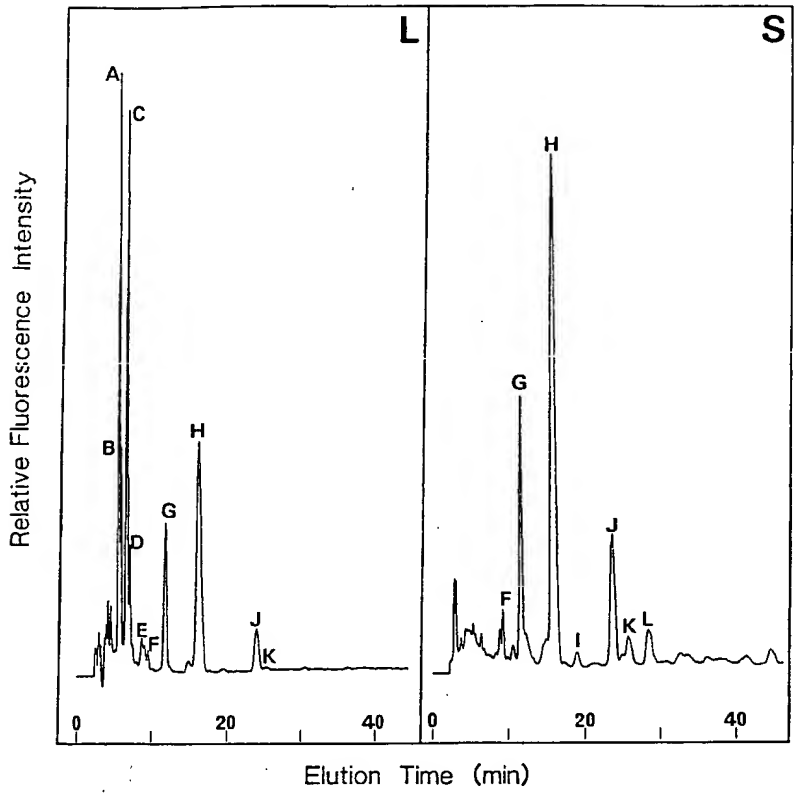
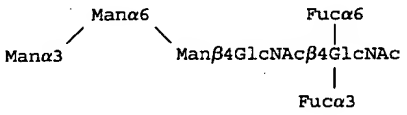


FIG. 2. **HPLC profiles of PA-oligosaccharides derived from the lysate and supernatant IgGs.** The IgGs were proteolyzed with trypsin/chymotrypsin, and the oligosaccharides were released with glycoamidase A. The oligosaccharides were pyridylaminated and subjected to separation on the ODS-silica column under the conditions described in the text. *L*, from lysate; *S*, from supernatant.

known standards. Furthermore, the unknown oligosaccharide eluted at a different position when coinjected with the known standard, 000.1F. Subsequent analysis indicated the elution position to be the same as the oligosaccharide M3.3F. Since the fucose residue α1,3-linked to the GlcNAc-1 cannot be removed by any of the α-fucosidases we have tested, its removal was carried out chemically with 13.5 M trifluoroacetic acid at 20 °C for 20 h (34). The de-α1,6- and de-α1,3-fucosylated oligosaccharide G coeluted with a $Man_3GlcNAc_2$ oligosaccharide, Man3.3 (Fig. 3), suggesting that the structure of oligosaccharide G is as follows,



STRUCTURE I

The structure of oligosaccharide G was further confirmed by ^1H NMR. The chemical shift values for the structural reporter groups of oligosaccharide G are summarized in Table II. Also shown are three reference compounds: PLA-9 from honeybee venom phospholipase A_2 having an α1,3-Fuc-linked to the GlcNAc-1 (14); IgG-E, an *N*-acetyllactosamine-type oligosaccharide from human IgG, having an α1,6-Fuc linked to the GlcNAc-1 (35); and BG60-B from Bermuda grass antigen BG60, having α1,3-Fuc linked to the GlcNAc-1 (36). The existence of the H-1 and H-2 chemical shifts for Man-3 (4.738 and 4.23 ppm) and Man-4′ (4.908 and 3.97 ppm) at 60 °C were assigned as noted for oligosaccharide G. Because of limited availability of oligosaccharide G, unambiguous assignment of H-1 and H-2 chemical shifts to Man-A or Man-4 were very difficult. The signals at 5.096 ppm (H-1) and 4.059 ppm (H-2) at 23 °C were tentatively assigned to those of Man-A. Although this signal may also be assigned to that of Man-4, the HPLC data clearly indicated that peak G, after removal of the α1,6-linked Fuc residue, is not identical to BG60-B. Thus the presence of Man-4 was ruled out.

The chemical shifts of the anomeric and methyl protons of oligosaccharide G clearly show the existence of two different Fuc residues. The values of 5.055 and 1.182 ppm (at 23 °C) are essentially the same as those of α1,3-Fuc in PLA-9 (5.052 and 1.20 ppm) and in BG60-B (5.057 and 1.20 ppm). The other chemical shift values of 4.863 ppm (H-1) at 60 °C, 3.978 ppm (H-5) and 1.194 ppm (CH_3) at 23 °C are very close to those for α1,6-Fuc linked to the GlcNAc-1, as seen in IgG-E. In addition, if there is the α1,3-Fuc on the GlcNAc-1, the H-2 of the GlcNAc-1 will shift from 4.32 to 4.48 ppm. By contrast, the α1,6-Fuc shows little influence on the H-2 of GlcNAc-1 (37). In oligosaccharide G, the observed chemical shift of the H-2 (4.460 ppm) for GlcNAc-1 indicates the α1,3-Fuc is linked to the GlcNAc-1. Thus all the chemical shift data are consistent with the structural assignment by the two-dimensional mapping technique. Moreover, ESI mass spectroscopic data (Fig. 4) are also in agreement with the structure for oligosaccharide G as shown above and in Table II.

Comparison of HPLC Oligosaccharide Profiles in the Presence and Absence of Coexpressed BiP—Purification of multiple IgG lysate samples from cells infected with JPLH alone provided sufficient oligosaccharide for reverse phase HPLC analysis on the ODS-silica column. A representative comparison of the oligosaccharide peak areas obtained in the presence and absence of BiP for both supernatant and lysate IgG samples is shown in Table III.

DISCUSSION

A comprehensive glycosylation profile, including intracellular and extracellular glycoforms, has been generated for heterologous IgG obtained from the efficient secreting insect cell host, *T. ni* (TN-5B1-4, High Five). The profile of *N*-glycans obtained from the culture medium indicates that High Five cells are capable of secreting complex-type glycoforms. The

TABLE I
The proposed structures of PA-oligosaccharides obtained from supernatant and lysate in insect cells

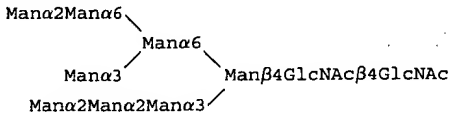
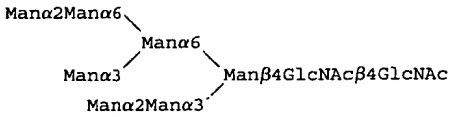
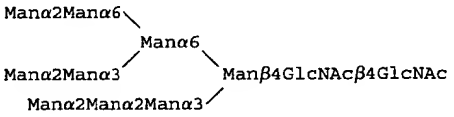
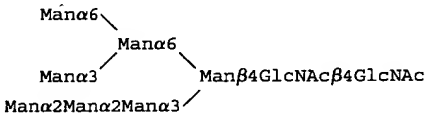
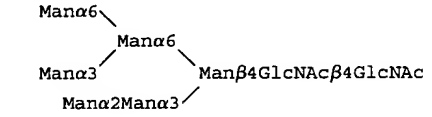
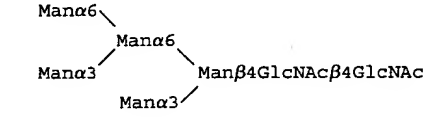
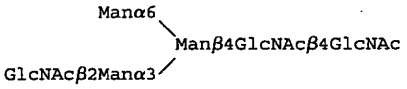
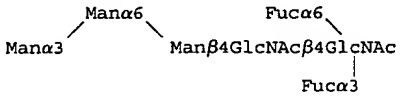
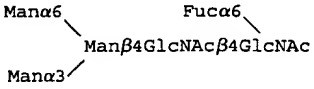
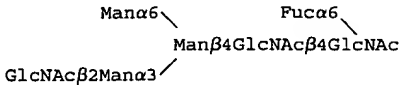
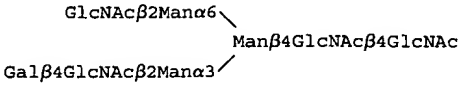
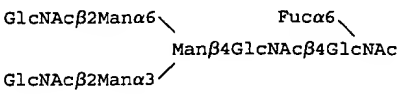
Peaks code no. (G.U.)[a]	Observed G.U. ODS, amide	Structures	Mol % Supernatant	Mol % Lysate
A M8.1 (4.9, 9.0)	5.1, 8.7			12.6
B1 M7.2 (5.1, 8.1)	5.3, 7.8			9.9
B2 M9.1 (5.2, 9.7)	5.3, 9.5			3.0
C M7.1 (5.8, 8.0)	6.0, 7.8			19.2
D M6.1 (6.1, 7.1)	6.3, 6.9			2.6
E M5.1 (7.2, 6.2)	7.1, 6.0			2.0
F 100.2 (7.4, 4.7)	7.3, 4.6		1.6	1.2
G New	8.5, 5.5		18.0	11.8
H1 010.1 (10.2, 4.7)	9.9, 4.6		16.7	9.5
H2 110.2 (10.2, 5.1)	9.9, 4.9		25.4	14.2
H3 200.3 (9.6, 6.1)	9.9, 5.8		14.2	8.1
J 210.1 (12.3, 5.5)	11.8, 5.3		16.0	5.6

TABLE I—*continued*

Peaks code no. (G.U.)	Observed G.U. ODS, amide	Structures	Mol % Supernatant	Mol % Lysate
K 110.1 (12.7, 5.1)	12.5, 5.0	GlcNAcβ2Manα6\ Fucα6\ Manβ4GlcNAcβ4GlcNAc Manα3/	2.7	0.3
L 210.2 (13.1, 6.3)	13.3, 6.4	Galβ4GlcNAcβ2Manα6\ Fucα6\ Manβ4GlcNAcβ4GlcNAc GlcNAcβ2Manα3/	5.4	

[a] Elution positions expressed in glucose unit, in the order of ODS, amide.

complex-type *N*-glycans (H-3, J, L) represent a significant fraction, over 35%, of the total secreted glycoforms although the structures were not observed to include sialic acid. Hybrid structures (F, H-2, K) represent an additional 30% of the glycoforms, with the remaining secreted structures principally in the paucimannosidic form (G, H-1). From studies of influenza hemagglutinin expressed in Sf9 cells, it was concluded that GlcNAc-TII and other glycosyltransferase enzymes are inherently absent in Sf9 cells (38). In contrast, *T. ni* cells are equipped with considerable levels of processing enzymes capable of producing complex-type structures. Baculovirus-infected *T. ni* larvae were also reported to secrete human placental alkaline phosphatase containing complex oligosaccharides including sialic acids in lectin blot studies (16). The High Five cells from *T. ni* appear to include some but not all of these processing enzymes.

The ratios of complex, hybrid, and paucimannosidic glycoforms in the secreted IgG are not unlike those obtained at the Asn^{25} site of interferon-γ secreted from Ea-4 cells (22). In addition, the current results indicate the presence of difucosylated *N*-glycans. The α1,3-linked fucose has been observed in honeybee venom PLA_2 and membrane glycoproteins of insects (5, 14) but never before in a heterologous protein from insect cells. This moiety may represent an important carbohydrate component of food, animal, and plant allergens (6, 39). Fucose α1,3-linked to the innermost GlcNAc was shown to be important for IgE and IgG binding to the honeybee allergen, PLA_2 (14, 40). This α1,3-fucose linkage is also a common structural feature of the oligosaccharides from the plant allergen BG60 of Bermuda grass (36).

A comparison of the carbohydrate structures from IgG_{2a} secreted from insect cells with those of the recombinant IgG_4 secreted from NS/0 mouse myeloma cells is also informative (41). While the proportion of complex structures obtained from NS/0 was higher (46 *versus* 35%), a number of the complex and hybrid structures obtained from the two sources were similar. The major difference in the structures obtained from the two sources was the presence of paucimannosidic *N*-glycans and the α1,3-linked fucose moiety from the secreted product of insect cells. No oligosaccharide structures containing sialic acids or bisecting GlcNAc were observed attached to *N*-glycans from the IgG of the murine NS/0 (41) or insect cells in this study. Some sialylation is common for most mammalian-derived *N*-glycans from IgG, although the level of sialylation and galactosylation can depend on the particular IgG considered (42–45). The sialylation of an *N*-linked oligosaccharide from human chimeric IgG_3 produced in Chinese hamster ovary cells increased from 5 to 75% by mutating amino acid 243 from phenylalanine to alanine (46). Consequently, the lack of sialic acid containing oligosaccharides observed in the current study may be a consequence of the structural characteristics of the particular IgG considered. However, it seems more likely that

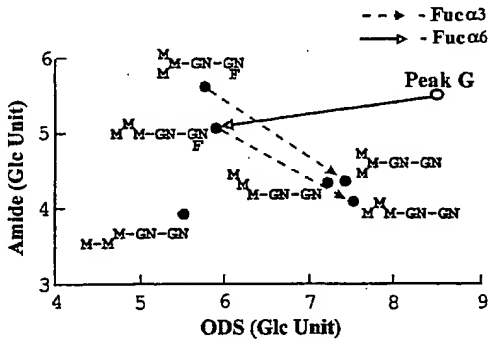
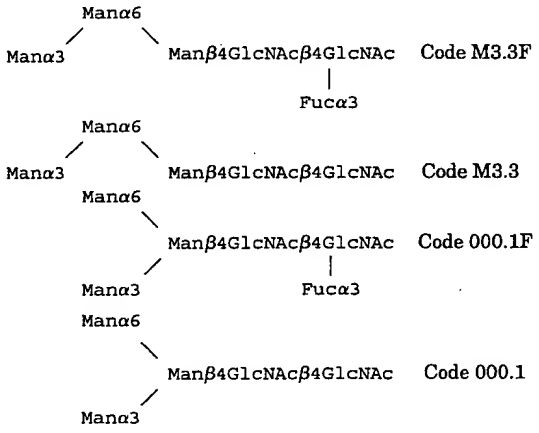


FIG. 3. **Structural identification of oligosaccharide G by two-dimensional mapping.** The coordinates of oligosaccharide G were superimposed with the coordinates of the reference compounds on the map. The *arrows* indicate the direction of changes in the coordinates: →, α1–6-fucosidase; - - →, chemical defucosylation. Chromatographic conditions are described in the text.



the absence of sialic acid is a consequence of the host processing characteristics. Recent reports of *N*-linked oligosaccharides from homologous and heterologous glycoproteins produced by several other insect cell lines showed a total absence of sialic acids (6, 22).

In contrast to the secreted glycoforms, the intracellular *N*-glycans from insect cells include more than 50% high mannose-type structures (A-E). The high level of high mannose-type structures from intracellular sources indicates that significantly less oligosaccharide processing occurred for many of the intracellular immunoglobulins. These intracellular immunoglobulins may not reach the compartments in which carbohydrate trimming takes place. This may be due to the endoplasmic reticulum or Golgi retention of some immunoglobulins (47) or slow secretory pathway processing in the baculovirus-infected cells (48). The baculovirus infection or cellular factors may limit or inhibit the transport of recombinant proteins

TABLE II
NMR data of oligosaccharide G and related oligosaccharides (measured at 23 and 60 °C)

Signals[a]	Compounds,[b] Residues	Peak G	PLA-9	IgG-E	BG60-B
		M F /\ \ M M-N-N A 3 / F	4' M \ M-N-N / / N-M F 4	N-M F \ \ M-N-N / 2 1 N-M	M \ M-N-N / / M F
H-1	GlcNAc-2	(4.683)	4.604	4.710	4.596
	Man-3	(4.738)		(4.749)	4.857
	Man-4		5.105	5.106	5.112
	Man-A	5.096 (5.113)			
	Man-4'	(4.908)	4.922	4.909 (4.899)	4.910
	Fucα(1,3)	5.055 (5.052)	5.052		5.057
	Fucα(1,6)	(4.863)		(4.850)	
H-2	GlcNAc-1	4.460	4.48[c]		
	Man-3	(4.23)[c]	4.246		4.256
	Man-4		4.167		4.034
	Man-A	4.059 (4.07)[c]			
	Man-4'	(3.97)[c]	3.983		3.974
H-5	Fucα(1,3)	4.242	4.25[c]		4.22[c]
	Fucα(1,6)	3.978		4.03	
CH3	Fucα(1,3)	1.182 (1.189)	1.20[c]		1.20[c]
	Fucα(1,6)	1.194 (1.189)		1.175	

[a] The values in parentheses were obtained at 60 °C. Chemical shifts were calculated with reference to internal acetone signal (2.216 ppm at 23 °C; 2.213 ppm at 60 °C).
[b] The numerals indicate residue numbers. For clarity, each residue number is marked only once.
[c] The assignments were with homonuclear Hartman-Hahn spectroscopy measured at 60 °C.

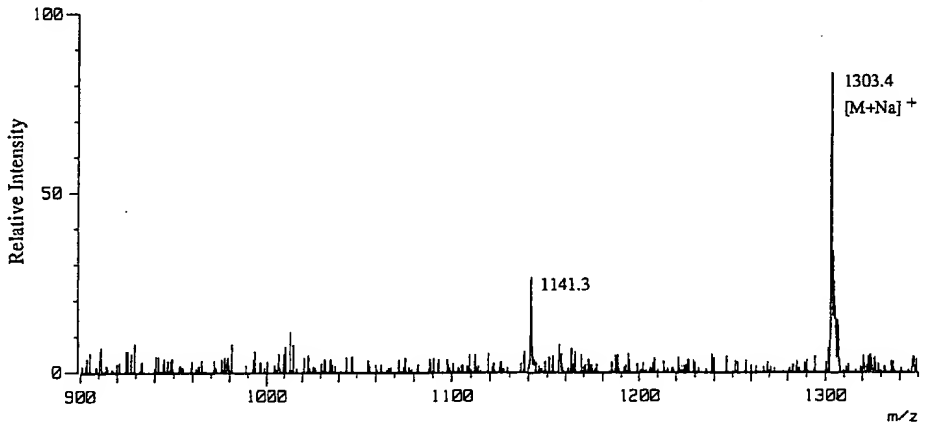


FIG. 4. **ESI-mass spectrogram of peak G.**

TABLE III
Comparison of relative percentages of N-glycan peaks determined from ODS-silica analysis of IgG purified from insect cell cultures infected with JPLH alone or coinfected with the viruses JPLH and AcBB-BiP

PEAK	Lysate		Supernatant	
	JPLH	JPLH + BiP	JPLH	JPLH + BiP
	mol %		*mol %*	
A	14.5	12.6	0.5	—
B	11.2	12.9	0.9	—
C	19.0	19.2	1.4	—
D	13.9	2.6	1.7	—
E	5.6	2.0	0.7	—
F	4.0	1.2	0.7	1.6
G	8.4	11.8	21.8	18.0
H	23.4	31.8	59.3	56.3
J	0.0	5.6	11.8	16.0
K	0.0	0.3	1.2	2.7
L	—	—	—	5.4
Total	100.0	100.0	100.0	100.0

through the secretory pathway. The levels of intracellular oligosaccharide processing enzymes could also affect the distribution of intracellular glycoforms (18, 20).

A comparison of the oligosaccharide peaks for intracellular and secreted IgG in the presence and absence of BiP indicates that the coexpression of the chaperone does not substantially alter the oligosaccharide profiles. In the absence of BiP, the level of intracellular high mannose glycoforms was still greater than 50%. The secreted glycoforms include few high mannose sugars and a significant content of more completely processed oligosaccharide forms. These similarities in the presence and absence of BiP suggest that the heterologous BiP was not the cause of the observed structural differences in intracellular and secreted glycoforms.

A putative pathway for oligosaccharide processing in the *T. ni* cells can be proposed based on the intracellular and extracellular structural information of this study and prior analyses (Fig. 5). The initial mammalian endoplasmic reticulum pathway, including trimming of the terminal glucose and mannose residues, seems operative. The trimming process appears to follow a linear pathway with the exception of two different forms of the $Man_7GlcNAc_2$ (M7.2 and M7.1), also observed for native insect glycoproteins (7) and IgG_4 from NS/0 cells (41). The presence of two M7 forms suggests the possibility of an alternative processing pathway (7) such as one that yields the M7.2 oligosaccharide through the action of endo-α-mannosi-

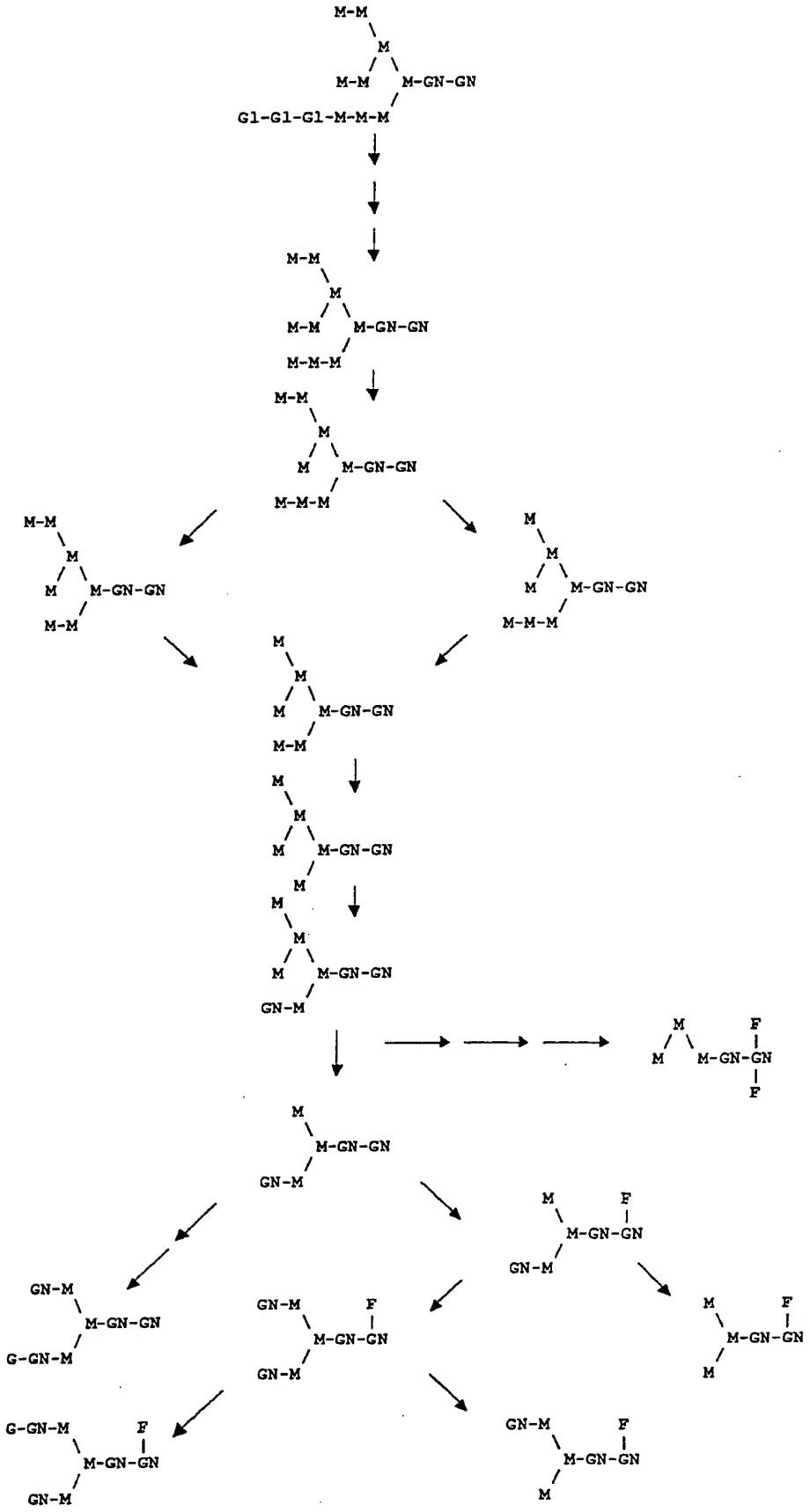


FIG. 5. *N*-Glycan processing pathway in *T. ni* insect cells.

dase. This enzyme, widely distributed in mammalian cells (49), has not yet been described in insect cells. Following cleavage of the mannose residues, GlcNAc is added to the α1,3 branch of $Man_5GlcNAc_2$ by GlcNAc TI (17). However, this may be a short lived intermediate quickly processed by α-Man II (50) since $GlcNAc_1Man_5GlcNAc_2$ was not observed in the *T. ni* cell lysate. At the $GlcNAc_1Man_3GlcNAc_2$ oligosaccharide, several branching steps in the *N*-glycan processing pathway appear to be possible in insect cells. Complex glycoforms can be generated which include terminal GlcNAc and Gal residues in the presence or absence of attached α1,6-linked fucose.

Insect cell processing diverges from mammalian cell processing in the pathways leading to the paucimannosidic structures. The critical step is likely to be cleavage of the GlcNAc from the α1,3-Man branch by β-*N*-acetylglucosaminidase localized in the Golgi apparatus (50). Another significant difference in insect cell processing is the α1,3-linked addition of fucose, also observed in the processing pathway of plants (51).

Clearly, differences exist in the carbohydrate structures of glycoproteins obtained from insect cells as compared with mammalian derived glycoproteins. However, many similarities exist as well, especially for glycoproteins secreted from cell lines such as *T. ni* (TN-5B1-4). Even so, it may still be desirable to modify glycoprotein processing even further to mimic more closely mammalian glycoforms. Wagner *et al.* (38) reported that coexpression of the GlcNAc T1 enhanced the level of oligosaccharides with GlcNAc on the α1,3-Man branch and Jarvis and Finn (52) reported that expression of β1,4-Gal T added Gal to the baculovirus glycoprotein 64 protein. The addition of chemical agents inhibiting oligosaccharide processing is another approach to alter glycoprotein processing. In this way, it may be possible to achieve an oligosaccharide profile that is even more reflective of the one obtained from mammalian cell sources.

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